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Interactions between Papillomavirus L1 and L2 Capsid Proteins

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The human papillomavirus (HPV) capsid consists of 360 copies of the major capsid protein, L1, arranged as 72 pentamers on a T=7 icosahedral lattice, with substoichiometric amounts of the minor capsid protein, L2. In order to understand the arrangement of L2 within the HPV virion, we have defined and biochemically characterized a domain of L2 that interacts with L1 pentamers. We utilized an in vivo binding assay involving the coexpression of recombinant HPV type 11 (HPV11) L1 and HPV11 glutathione *S*-transferase (GST) L2 fusion proteins in *Escherichia coli*. In this system, L1 forms pentamers, GST=L2 associates with these pentamers, and L1+L2 complexes are subsequently isolated by using the GST tag on L2. The stoichiometry of L1:L2 in purified L1+L2 complexes was 5:1, indicating that a single molecule of L2 interacts with an L1 pentamer. Coexpression of HPV11 L1 with deletion mutants of HPV11 L2 defined an L1-binding domain contained within amino acids 396 to 439 near the carboxy terminus of L2. L2 proteins from eight different human and animal papillomavirus serotypes were tested for their ability to interact with HPV11 L1. This analysis targeted a hydrophobic region within the L1-binding domain of L2 as critical for L1 binding. Introduction of negative charges into this hydrophobic region by site-directed mutagenesis disrupted L1 binding. L1-L2 interactions were not significantly disrupted by treatment with high salt concentrations (2 M NaCl), weak detergents, and urea concentrations of up to 2 M, further indicating that L1 binding by this domain is mediated by strong hydrophobic interactions. L1+L2 protein complexes were able to form virus-like particles in vitro at pH 5.2 and also at pH 6.8, a pH that is nonpermissive for assembly of L1 protein alone. Thus, L1/L2 interactions are primarily hydrophobic, encompass a relatively short stretch of amino acids, and have significant effects upon in vitro assembly.

Papillomaviruses are small, nonenveloped, double-stranded DNA viruses. These viruses are pathogens of epithelial surfaces and cause a variety of proliferating lesions in humans and animals. Infection by high-risk subtypes of human papillomavirus (HPV) such as HPV type 16 (HPV16) and HPV18 is directly related to the subsequent development of cervical cancer (4, 38, 39). Papillomavirus capsids are ca. 600 Å in diameter and composed of 72 pentameric capsomeres arranged in a T=7 icosahedral lattice (2, 7, 50). Each capsomere contains five monomers of the 55-kDa major capsid protein, L1. The capsid also contains ca. 12 copies of the 74-kDa L2 minor capsid protein, possibly associated with the 12 pentavalent capsomeres (13, 50). Expression of recombinant L1 or L1+L2 in a variety of expression systems results in the self-assembly of virus-like particles (VLPs) that approximate the structure of native virions (22, 30, 31, 44, 46, 55). The structure of "small," T=1 VLPs assembled from HPV16 L1 expressed in *Escherichia coli* (6) has recently been determined at a 3.5-Å resolution, providing insights into the conformation of neutralizing epitopes, potential receptor binding sites, and the nature of intercapsomeric contacts (7). This structure has led to an atomic model of full-sized T=7 papillomavirus capsids (35) in which L1 pentamers interact with one another via flexible carboxy-terminal arms in a similar fashion to that observed for capsomere interactions in polyomaviruses (32).

The structural relationship of L2 to L1 within the papillomavirus virion is unknown. Three-dimensional reconstruction of cryoelectron micrographs of bovine papillomavirus (BPV) virions at a 9-Å resolution suggests that L2 associates with the 12 pentavalent capsomeres present on the virion (50). Some researchers have estimated that there are as many as 36 copies of L2 per virion (13). By analogy with the relationship of the polyomavirus minor capsid proteins VP2 and VP3, with the major structural protein, VP1 (8, 21), L2 may interact with residues in the central cavity of an L1 pentamer. The disassembly characteristics of HPV33 VLPs under various salt conditions indicates that L1-L2 interactions are noncovalent and have both salt-dependent and salt-independent contacts (45). Recently, two distinct L1-binding domains have been identified for BPV type 1 (BPV1) L2, both of which are required for encapsidation of the BPV1 genome (37). The involvement of L2 in genome encapsidation coupled with the DNA-binding properties of L2 (14, 34, 54) suggests that, within a virion, L2 forms contacts with the viral genome in addition to contacts with L1 pentamers. Another domain of L2 may be exposed at the virion surface, forming a specific epitope for immune recognition (15, 26, 27, 33). This surface-exposed region of L2 may also interact with a cellular receptor to facilitate uptake of virions (28).

In order to characterize the position of L2 within the papillomavirus capsid, we sought to define the domain(s) on HPV11 L2 that interact with L1 pentamers. We utilized a bacterial coexpression system in which L1+L2 complexes form in the bacteria and are then purified. By analyzing the ability of truncated and mutated forms of L2 to form complexes, we defined an L1-binding domain within a 44-amino-acid region

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TABLE 1. Primers used for constructing deletion and site-directed mutants of HPV11 L2

Plasmid	Primer sequence (5′ to 3′)[a]	
	Forward	Reverse
pXA/BN-HPV11L2/1–156	GGGGGATCCATGAAACCTAGGGCACGC	GGGGCGGCCGCATTTTGAAACACACTAGTGG
pXA/BN-HPV11L2/1–309	GGGGGATCCATGAAACCTAGGGCACGC	GGGGCGGCGGCTCCACTGCGTGTGTACATG
pXA/BN-HPV11L2/157–309	GGGGGATCCCCCCTGTTTACAGAACCG	GGGGCGGCGGCTCCACTGCGTGTGTACATG
pXA/BN-HPV11L2/157–455	GGGGGATCCCCCCTGTTTACAGAACCG	GGGGCGGCCGCCTAGGCCGCCACATCTG
pXA/BN-HPV11L2/313–455	CAAGGATCCGGTGCCCGCATACATTAT	GGGGCGGCCGCCTAGGCCGCCACATCTG
pXA/BN-HPV11L2/313–400	CAAGGATCCGGTGCCCGCATACATTAT	AATTCCGCGGCCGCTATGTCAGGCCCAGA
pXA/BN-HPV11L2/346–455	AATTCCGGATCCGATATTTATGCTGAA	GGGGCGGCCGCCTAGGCCGCCACATCTG
pXA/BN-HPV11L2/396–455	AATTCCGGATCCTCTGGGCCTGACATA	GGGGCGGCCGCCTAGGCCGCCACATCTG
pXA/BN-HPV11L2/346–439	AATTCCGGATCCGATATTTATGCTGAA	AATTCCGCGGCCGCTGCAAAGTACCATGAGG
pXA/BN-HPV11L2/396–439	AATTCCGGATCCTCTGGGCCTGACATA	AATTCCGCGGCCGCTGCAAAGTACCATGAGG
pXA/BN-HPV11L2/313–455/AL417418EE	CTGTAACTCCTG**AAGA**ACCTACAGGCCC	GGGCCTGTAGGT**TCTT**CAGGAGTTACAG
pXA/BN-HPV11L2/313–455/A417E	CTGTAACTCCTG**AA**TTACCTACAGGCCC	GGGCCTGTAGGTAA**TT**CAGGGTGTACAG
pXA/BN-HPV11L2/313–455/L418EE	CTGTAACTCCTGCT**GA**ACCTACAGGCCC	GGGCCTGTAGGT**TC**AGCAGGAGTTACAG
pXA/BN-HPV11L2/313–455/P413A	ACACCCTTTAGT**G**CTGTAACTCCTG	CAGGAGTTACAGCACTAAAGGGTGT
pXA/BN-HPV11L2/313–455/P416A	GTCCTGTAACT**G**CTGCTTTACCTAC	GTAGGTAAAGCAGCAGTTACAGGAC
pXA/BN-HPV11L2/313–455/P419A	ACTCCTGCTTTA**G**CTACAGGCCCTG	CAGGGCCTGTAGCTAAAGCAGGAGT

[a] Restriction sites within the primer are underlined; mutations within the primer are indicated in boldface.

near the carboxy terminus of L2 and identified a hydrophobic region within this domain that facilitated L1 binding. Purified L1+L2 complexes also permitted a biochemical analysis of L1-L2 interactions and investigations into the specific effects of the minor capsid protein on in vitro assembly of VLPs.

MATERIALS AND METHODS

Cloning and construction of deletion and site-directed mutants. Full-length HPV11 L1 DNA sequence was obtained by PCR amplification from plasmid pVL11L1 (44) with the forward primer 5′-GCCGCGAAGCTTCATATGTGG CGGCCTAGCGCAG, containing an *Nde*I restriction enzyme site at the initiator methionine codon, and the reverse primer, 5′-GGGCCTGGATCCAG ATCTCACAACACACACTGACACAC. The PCR-amplified fragment was subcloned into a PCR II vector (Invitrogen) by using manufacturer's protocols. An *Nde*I/*Bst*XI fragment encompassing the amplified sequences was excised from this intermediate vector, purified, and then ligated to similarly digested pET17b vector (Novagen) to generate pET17b-HPV11 L1. The pXA/BN-based vectors, used for expressing HPV11 GST=L2 fusion proteins and all other GST=L2 fusion protein derivatives described here, were engineered from the original pAC vector described by Chen et al. (8) to incorporate the multiple cloning site of pGEX-4T-2 (Pharmacia). Originally, full-length HPV11 L2 DNA was obtained by PCR amplification from a PCR II vector (Invitrogen) containing full-length HPV11 L2 DNA (PCRII/L2) with a forward primer, 5′-GGGGGATCCATGAAACCTAGGGCACGC, containing a *Bam*HI restriction site at the 5′ terminus and a reverse primer, 5′-GGGGCGGCCGCCTAGGCCGCCACATC TG, containing a *Not*I restriction site at the 3′ terminus. The PCR-amplified fragment was subcloned into a PCR II vector, and then a *Bam*HI/*Not*I fragment excised from this intermediate vector was ligated to similarly digested pGEX-4T-2. The pGEX-4T-2 vector containing L2 was used as a source of *Bam*HI/*Not*I fragment to subclone into pXA/BN to generate pXA/BN-HPV11 L2. A similar strategy was used to construct the initial deletion mutants of HPV11 L2 encompassing amino acids 1 to 156, 1 to 309, 157 to 309, 157 to 455, and 313 to 455. The primer pairs used for PCR amplification of these and subsequent deletion mutants are given in Table 1. For subsequent deletion mutants, the HPV11 L2 DNA was amplified from pXA/BN-HPV11 L2 and subcloned into pCR2.1-TOPO vector (Invitrogen) according to the manufacturer's instructions. *Bam*HI/*Not*I fragments encompassing the amplified sequences were excised from these intermediate vectors and subcloned into pXA/BN. This strategy was also used to clone sequences corresponding to the carboxy termini of various papillomavirus L2s, with the exception of BPV1 L2, which was cloned as a *Bam*HI/*Xho*I fragment. These full-length L2 sequences were aligned with the full-length sequence of HPV11 L2 by using Vector NTI AlignX software (InforMax) to define candidate amino acids for incorporation into each construct (Table 2). Overlap extension PCR with paired mutagenic primers (24, 52) was used for the site-directed mutagenesis of specific HPV11 L2 amino acids. The sequence of these mutagenic primers is given in Table 1; the forward and reverse outside primers used to amplify a fragment containing the mutated sequence were 5′-GGGCT GGCAAGCCACGTTTGGTG and 5′-AATTCCAGATCTATACACTCCGCT ATCGC, respectively. Cloning of the amplified sequences into pXA/BN was performed as outlined above. The sequence of all DNA subjected to PCR amplification was verified by sequencing.

Expression and purification of L1+L2 complexes. Plasmid pET17b-HPV11 L1 was cotransformed with the appropriate pXA/BN-based L2 expression plas-

TABLE 2. Other papillomavirus L2 expression constructs used in this study

Virus	L2 amino acids incorporated	NCBI reference no.[a]	Primer sequence (5′ to 3′)[b]	
			Forward	Reverse
HPV6b	314–459	NC_001355	AATTCCGGATCCGGGGCCCGCATTCATTATTTTTA	AATTCCGCGGCCGCCTAGGCCGCCACATCTG
HPV16	321–473	NC_001526	AATTCCGGATCCGGTGCTAAGGTACATTATTATTA	AATTCCGCGGCCGCCTAGGCAGCCAAAGAGAC
HPV33	319–467	NC_001528	AATTCCGGATCCGGAGCTAGAATACATTATTATC	AATTCCGCGGCCGCCTAGGCCGCCACACGGAC
HPV1a	347–507	NC_001356	AATTCCGGATCCGGGCCACAAAGCCATTTTTAC	AATTCCGCGGCCGCTTAAAAAAAAAAAATGTTTGCG
HPV5	376–518[c]	NC_001531	AATCCGGATCCGGGTCGCAAGTCCATTTTTAC	AATTCCGCGGCCGCTCACAAATATTTTCTT
HPV12	360–518	NC_001577	AATTCCGGATCCGGATCACAGGTTCATTTTTATAG	AATTCCGCGGCCGCTCACAAATATTTTCTT
COPV1	360–513	NC_001619	AATTCCGGATCCGGGCCACAAAGCCATTTTTAC	AATTCCGCGGCCGCTTAAAAAAAAAAAATGTTTGCG
BPV1	316–469	NC_001522	AATTCCGGATCCGGACCACAGCTACATGTCAGG	CTCGAGTTAGGCATGTTTCCGTTTTTTTCGTTTC

[a] Sequences are the same as those given in the NCBI reference with the exception of the HPV5 isolate. The relevant amino acid changes within the putative L1 binding domain of HPV5 are indicated on Fig. 5.

[b] Restriction sites within the primer are underlined.

[c] Although primers were designed such that HPV5 amino acids 360 to 518 would be incorporated, only amino acids 376 to 518 are present due to the presence of a cryptic *Bam*HI site at position 5472 in the HPV5 isolate used for PCR amplification.

mid into the bacterial host BL21(DE3). Typically, a single isolated colony of cotransformed bacteria was inoculated into 5 ml of 2× yeast-tryptone medium supplemented with ampicillin (100 μg/ml) and chloramphenicol (40 μg/ml) and grown overnight at 37°C. Fresh medium (5 ml) was inoculated with 0.1 ml of this overnight culture and allowed to grow 5 to 6 h at 37°C. This culture was used to inoculate larger-scale cultures (one 5-ml starter culture per liter) which were subsequently grown at 37°C to an optical density at 600 nm of ~0.2. The cultures were then induced with 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and grown at 37°C for 5 to 6 h or cooled to 25°C, induced with 0.2 mM IPTG and grown overnight at 25°C. Cell pellets were resuspended in lysis buffer (40 mM Tris-Cl [pH 8.0], 0.2 M NaCl, 5% glycerol, 1 mM EDTA, 5 mM dithiothreitol [DTT]) supplemented with protease inhibitors (5 μg of pepstatin/ml, 2 μg of leupeptin/ml, 2 mM phenylmethylsulfonyl fluoride). The following steps were carried out at 4°C. Cells were lysed by the addition of 0.5 mg of lysozyme/ml, followed by incubation for 20 min, followed by the addition of 0.1% deoxycholate and incubation for an additional 10 min. The lysates were then treated with 10 U of DNase I/ml in the presence of 5 mM $MgCl_2$ for 30 min. To reduce the viscosity of the DNase-treated lysate, the lysates were briefly sonicated. The sonicated lysates were then centrifuged at 17,000 × *g* for 1 h, and the supernatant was passed over a 10-ml bed volume glutathione-Sepharose column (Pharmacia). The columns were washed with buffer L (40 mM Tris-Cl [pH 8.0], 0.2 M NaCl, 1 mM EDTA, 2 mM DTT) until no protein was detectable in the wash (~20 bed volumes). To purify L1+GST=L2 complexes, bound proteins were eluted with 10 mM reduced gluthathione in buffer L. To purify L1+L2(313-455) complexes with the GST tag removed, the columns were washed with 1.5 bed volumes of buffer L without DTT and then treated with ~1 NIH unit of thrombin per 100 μg of protein in 1.5 bed volumes of buffer L without DTT for at least 1 h at room temperature. Thrombin-treated columns were then eluted with buffer L without DTT, and the DTT concentration of the eluate was adjusted to 10 mM by the addition of 1 M DTT. The eluates were concentrated by using Centriplus concentrators (Amicon). To further purify L1+L2 complexes from contaminating bacterial proteins, free L2, and L2 degradation and/or premature termination products, eluted proteins were subjected to size exclusion fast-pressure liquid chromatography (FPLC) by using a HiLoad Superdex 200 gel filtration column (Pharmacia). FPLC fractions containing L1+L2 complexes were pooled and further concentrated as outlined above. Protein complexes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie blue staining or immunoblotting (44). For all coexpressions, samples of the soluble fraction of the bacterial lysate prior to glutathione-Sepharose chromatography were verified by immunoblot analysis for the presence of L1. For densitometric analysis on Coomassie blue-stained gels, images were captured by using a Fluor-S MultiImager (Bio-Rad) with Quantity One software (Bio-Rad) and quantitated by using ImageQuant software (Molecular Dynamics).

Disruption of L1+L2 complexes. A complex of HPV11 L1 bound to HPV11 GST=L2(313-455) was immobilized on glutathione-Sepharose beads. Equal fractions of these beads were subjected to the buffer treatments described in the legend to Fig. 8 for 1 h at room temperature. Treated beads were then pelleted by centrifugation, and 50-μl samples of the supernatants were analyzed by SDS-PAGE and immunoblotting for the presence of L1. Densitometric analysis on Coomassie blue-stained gels was performed as described above in three independent experiments.

Assembly conditions and electron microscopy. Purified proteins (100 μg/ml) were dialyzed by microdialysis into assembly buffer that did not contain disulfide reducing agents (40 mM HEPES [pH 6.8], 0.5 M NaCl or 40 mM sodium citrate [pH 5.2], 0.5 M NaCl) at room temperature for 1 h (6). Samples were absorbed onto glow-discharged Formvar-carbon-coated 400 mesh copper grids and stained with 2% uranyl acetate. Specimens were analyzed by transmission electron microscopy by using a CM10 electron microscope (Phillips Electronic Instruments, Inc.) operating at 80 kV.

RESULTS

Bacterial coexpression of L1 and L2. Initially, HPV11 L1-L2 interactions were studied by using an in vitro binding assay. HPV11 L1 pentamers and HPV11 L2 GST fusion proteins (GST=L2) were purified after their individual expression in *E. coli*. GST=L2 was immobilized on glutathione-Sepharose, purified L1 was incubated with the protein bound to the column, and eluates from the column were analyzed for the presence of L1. We were unable to detect binding of L1 to GST=L2 with this assay (data not shown). We considered that (i) GST=L2, though soluble, was purified in a conformation that impaired its ability to bind L1, or (ii) L1 pentamers forming in the absence of L2 might adopt a conformation that excludes L2 from subsequent direct binding.

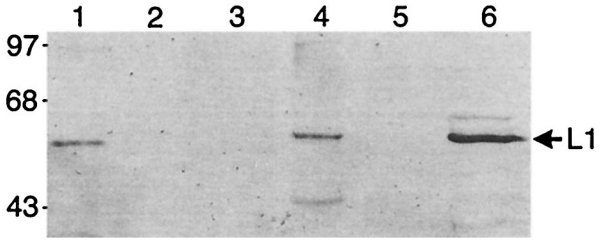

FIG. 1. HPV11 GST=L2 specifically binds HPV11 L1 in coexpressing cells. Samples from HPV11 L1 coexpressed with GST alone (lanes 1 to 3) or with HPV11 GST=L2 (lanes 4 to 6) were immunoblotted with α-L1 antisera. Lanes 1 and 4 contain 5 μl of the starting material prior to glutathione-Sepharose chromatography, lanes 2 and 5 contain 100 μl of the last wash prior to elution, and lanes 3 and 6 contain 10 μg of the reduced glutathione eluate. Molecular size markers in are indicated at the left in kilodaltons.

We then utilized a system in which L1 and L2 were expressed simultaneously. We adapted a bacterial coexpression system previously developed by Chen et al. for studying interactions between the major (VP1) and minor (VP2) structural proteins of mouse polyomavirus. In this system, the capsid proteins are coexpressed in *E. coli* from compatible plasmids carrying different selectable markers. Protein complexes form in the bacteria and are subsequently purified by glutathione-Sepharose chromatography by using a GST tag on the minor capsid protein (8).

HPV11 L1 and L2 were used as the homologous protein pair for the initial studies. HPV11 L2 was coexpressed as a GST fusion protein with HPV11 L1 in *E. coli* BL21(DE3). As shown in Fig. 1, HPV11 L1 coeluted with GST=L2 from the glutathione-Sepharose column when coexpressed with GST=L2 but not with GST alone. Samples of the soluble fraction of the bacterial lysate prior to glutathione-Sepharose chromatography (referred to as starting material in Fig. 1) and samples of the last column wash prior to elution were assayed for HPV11 L1. Using this bacterial coexpression system, up to 5 mg of partially purified L1+L2 complexes were obtained per liter of bacterial culture.

Stoichiometry of the HPV11 L1+L2 complex. Because the ratio of VP1:VP2 in the polyomavirus VP1+VP2 complex is 5:1, we anticipated that our L1+L2 complex would have a similar stoichiometry. Densitometric analysis of partially purified bacterial eluates yielded an L1/L2 ratio of ca. 2:1 (Fig. 2, lane 1). We reasoned that free GST=L2 present in these partially purified eluates would skew the L1:L2 stoichiometry. In addition, partially purified eluates, like those shown in Fig. 2, contain degradation and/or premature termination products that originate from the L2 fusion protein (as determined by anti-GST immunoblot analysis [data not shown]), as well as contaminating bacterial chaperone proteins GroEL and DnaK (data not shown). To remove unbound GST=L2 and chaperones, glutathione-Sepharose eluates of HPV11 L1 bound to HPV11 GST=L2(313-455) (Fig. 3) were subjected to size exclusion chromatography. The L1/L2 ratio in the resulting pu-

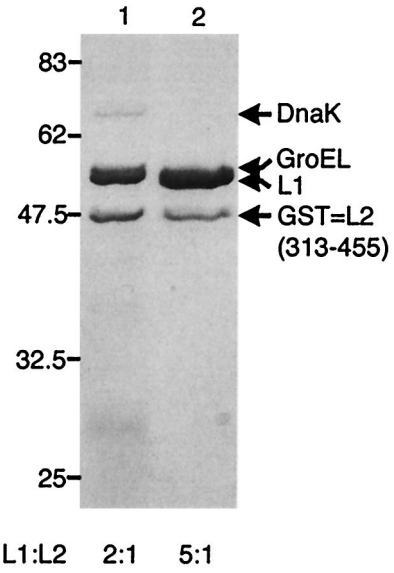

FIG. 2. L1/L2 stoichiometry in initial eluates and purified complexes. A total of 10 μg each of partially purified eluate (lane 1) or FPLC-purified L1+L2 complex (lane 2) from a coexpression of HPV11 L1 with HPV11 GST=L2(313-455) (Fig. 3) was separated by SDS-PAGE and stained with Coomassie blue, and then the ratio of L1 to L2, indicated below each lane, was determined by densitometry analysis. The positions of HPV11 L2 and HPV11 GST=L2(313-455) are indicated at the right, and molecular size markers are indicated at the left in kilodaltons.

rified complexes was ca. 5:1 (Fig. 2, lane 2). Thus, we conclude that a single molecule of L2 associates with a pentamer of L1, a ratio analogous to that observed in polyomavirus VP1-VP2 interactions (8). The increased L1/L2 ratio in the FPLC-purified L1+L2 complexes confirms that unassociated GST=L2 was present in partially purified eluates. Indeed, a broad eluting peak containing unassociated GST=L2 was detected in our FPLC analysis (data not shown).

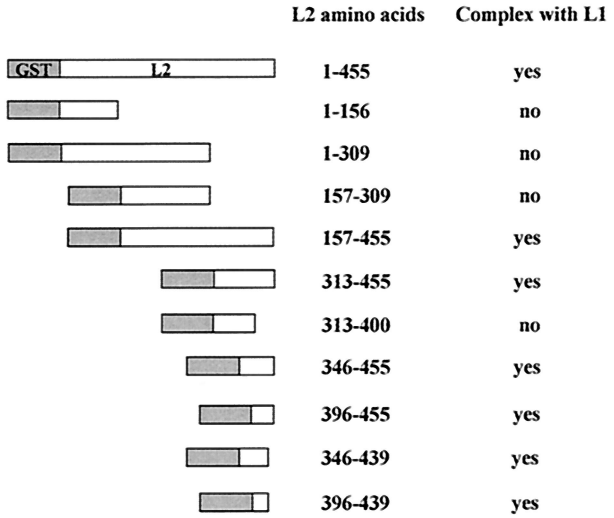

FIG. 3. HPV11 GST=L2 fusion proteins. White rectangles depict HPV11 L2 or deletion derivatives of HPV11 L2; gray rectangles depict GST fused to the amino terminus of L2 or L2 deletion derivatives. The L2 amino acids incorporated into each fusion protein are indicated beside each depiction. The ability of each fusion protein to form a complex with HPV11 L1 was determined by the ability to detect L1 in bacterial coexpression experiments (see the text for details and see Fig. 4 for examples).

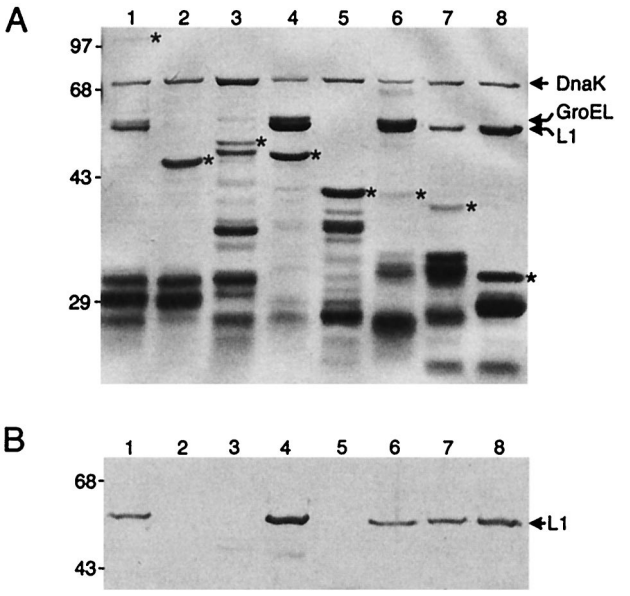

FIG. 4. Analysis of HPV11 L1 binding by using deletion derivatives of HPV11 L2. A total of 10 μg of reduced glutathione eluates was separated by SDS-PAGE and stained with Coomassie blue (A) or immunoblotted with α-L1 antisera (B). Lanes 1 through 8 in both panels correspond to coexpression of HPV11 L1 with HPV11 GST=L2, HPV11 GST=L2(1-156), HPV11 GST=L2(157-309), HPV11 GST=L2(313-455), HPV11 GST=L2(313-400), HPV11 GST=L2(346-455), HPV11 GST=L2(346-439), and HPV11 GST=L2(396-439). The asterisks in panel A represent the positions of the full-length fusion proteins; the position of HPV11 L1 and the bacterial chaperone proteins GroEL and DnaK are indicated at the right. Molecular size markers are indicated at the left of each panel in kilodaltons.

Identification of a carboxy-terminal L1-binding domain. The interaction of L1 with L2 in *E. coli* provides an assay for detecting domains of L2 affecting L1 binding. A series of deleted forms of HPV11 L2 (Fig. 3; the L2 amino acids present in each truncated derivative are designated and identify the construct) were analyzed by using the bacterial coexpression assay. For each combination, immunoblot analysis verified the presence of L1 in the initial bacterial extract. The ability of each L2 protein to bind HPV11 L1 was determined by the presence or absence of L1 in the eluate from the glutathione-Sepharose column (Fig. 4). This assay identified a single domain, contained within amino acids 396 through 439, near the carboxy terminus of HPV11 L2 that affected HPV11 L1 binding. This binding domain was distinct from the L2 nuclear localization signal previously identified between residues 440 and 445 (49).

L2 residues facilitating L1 binding. In order to identify specific residues within the 44-amino-acid domain of L2 affecting L1 binding, we analyzed the ability of HPV11 L1 to bind L2 proteins from eight different papillomavirus serotypes (Table 3). An alignment of the putative L1-binding domains of the L2 proteins used in this analysis is presented in Fig. 5. The carboxy-terminal third of each L2 was utilized in coexpression

TABLE 3. HPV11 L1 interactions with L2 proteins from other papillomaviruses

Virus	Host	Location of infection (level of risk)[a]	Supergroup[b]	L1/L2 ratio[c]	Interaction with HPV11 L1[d]
HPV11	Human	Genital tract (low)	A10	>1:1	Strong
HPV6b	Human	Genital tract (low)	A10	>1:1	Strong
HPV16	Human	Genital tract (high)	A9	>1:1	Strong
HPV33	Human	Genital tract (moderate)	A9	>1:1	Strong
HPV1a	Human	Skin	E1	ND	Undetectable
HPV5	Human	Skin	B1	<1:1	Weak
HPV12	Human	Skin	B1	<1:1	Weak
COPV1	Dog	Oral	E	<1:1	Weak
BPV1	Cow	Skin	C1	ND	Undetectable

[a] Risk of genital tract isolates for progression to cervical carcinoma.
[b] Supergroup designation is that defined in *Human Papillomaviruses* (1) based on a phylogenetic tree computed from a consensus primer region of L1.
[c] Relative amounts of L1 to the GST=L2 fusion proteins indicated with aterisks on Fig. 6A were determined by densitometric analysis of Coomassie blue-stained SDS-polyacrylamide gels.
[d] Strength of interaction with L1 is based on the ability to detect L1 by immunoblot analysis, as well as the relative amounts of L1 and L2. ND, not determined.

assays (Table 2). For each combination, immunoblot analysis verified the presence of L1 in the initial bacterial extract.

HPV11 L1 formed heterologous complexes with L2 proteins from other genital isolates (HPV6b, HPV16, and HPV33), from skin isolates (HPV5 and HPV12) and, surprisingly, from a nonhuman isolate, COPV1 (Fig. 6A, lanes 2 to 4, and Fig. 6B, lanes 6 to 8). HPV11 L1 was unable to form a complex with either HPV1a L2 or BPV1 L2 despite robust production of the L2 fusion protein in both cases (Fig. 6, lanes 5 and 9). The stoichiometry between L1 and the GST=L2 fusion protein (indicated by the asterisks on Fig. 6A) in the partially purified extracts of heterologous complexes formed with the genital isolates was greater than 1:1, as was the case for the homologous complex (Fig. 6A, lanes 1 to 4). This stoichiometry was not found in partially purified extracts of heterologous complexes formed with the skin isolates or with COPV1. Instead, the L1:L2 stoichiometry observed was less than 1:1 (Fig. 6A, lanes 6 to 8). One explanation for the differences in stoichiometry is that binding was weaker in the case of complexes formed between HPV11 L1 and L2 proteins from HPV5 and HPV12 and COPV1, and thus more unassociated L2 was detectable. Accordingly, we classified L1 binding by the genital isolates as strong, the skin isolates and COPV1 as weak, and HPV1a and BPV1 as undetectable (Table 3).

Inspection of the alignment in Fig. 5 does not reveal an obvious primary amino acid sequence unique to those L2 proteins capable of binding HPV11 L1. Instead, we noted that the sequence segment bounded by conserved proline residues at 413 and 419 of HPV11 L2 (boxed in Fig. 5) is predominantly hydrophobic without negatively charged residues in the L2 proteins classified as strong HPV11 L1 binders. In the case of weak binders, at least one negatively charged amino acid is present and in the case of both HPV1a and BPV1, where binding was undetectable, two negatively charged amino acids are present. Based on these observations, we hypothesized that disruption of this hydrophobic tract with negatively charged residues would disrupt HPV11 L1 binding. To test this hypothesis, alanine 417 and leucine 418 of HPV11 L2 were both changed to glutamines (AL417418EE), and the ability of this mutant L2 protein to bind L1 was examined in coexpression

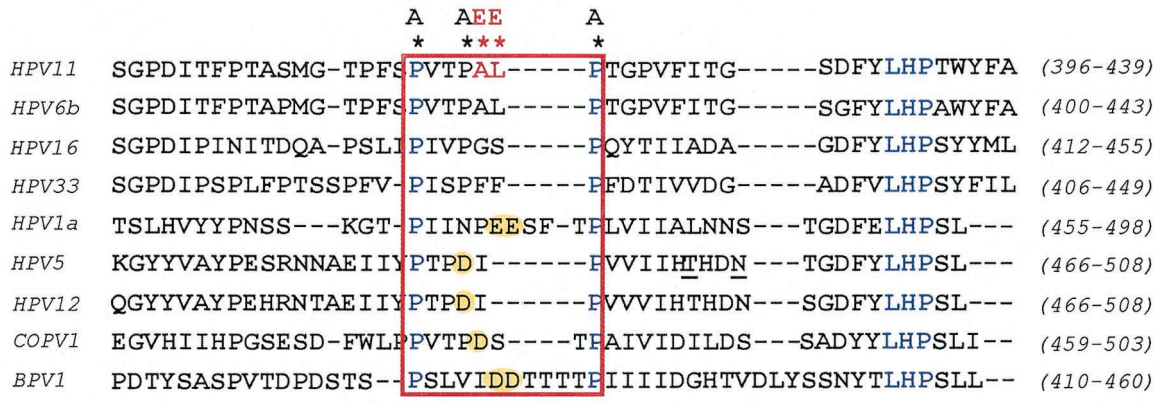

FIG. 5. Alignment of the putative L1-binding domains of selected L2 proteins. Alignments were generated with the assistance of Vector NTI AlignX software (InforMax). Conserved residues are in blue. Boxed residues indicate the hydrophobic region between the conserved prolines at 413 and 419 of HPV11 L2; negatively charged residues within this region are highlighted in yellow. Asterisks indicate HPV11 L2 amino acids subjected to site-directed mutagenesis with the change indicated above each asterisk. The combined alanine- and leucine-to-glutamine changes at 417 and 418 of HPV11 L2, indicated in red, severely disrupted L1 binding. Underlined residues in the HPV5 sequence are those that are different from the National Center for Biotechnology Information (NCBI) reference sequence (Table 2). Amino acids included in the alignment are indicated in brackets at the right.

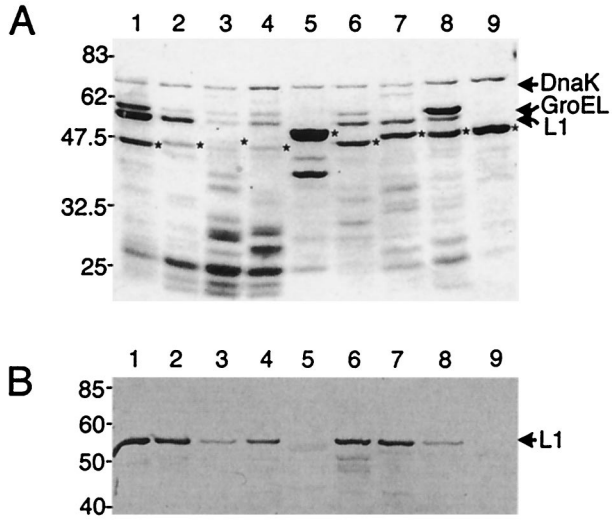

FIG. 6. HPV11 L1 forms heterologous complexes with other papillomavirus L2s. A total of 10 μg of reduced glutathione eluates were separated by SDS-PAGE and stained with Coomassie blue (A) or immunoblotted with α-L1 antisera (B). Lanes 1 through 8 in both panels correspond to coexpression of HPV11 L1 with HPV11 GST=L2(313-455), HPV6b GST=L2(314-459), HPV16 GST=L2(321-473), HPV33 GST=L2(319-467), HPV1a GST=L2(347-507), HPV5 GST=L2(376-518), HPV12 GST=L2(360-518), COPV1 GST=L2(360-513), and BPV1 GST=L2(316-469), respectively. The asterisks in panel A represent the positions of full-length fusion proteins; the position of HPV11 L1 is indicated at the right. Molecular size markers are indicated at the left of each panel in kilodaltons.

assays. As shown in Fig. 7 lane 2, the double mutant L2 protein AL417418EE was weakened in its ability to bind L1 with an L1/L2 ratio of 1:3 compared to 2:1 for wild-type L2 protein (lane 1). The A417E mutant L2 protein (lane 3) displayed a slight disruption in L1 binding (L1/L2 = 1:1), whereas the L418E mutant L2 protein (lane 4) displayed no disruption in HPV11 L1 binding (L1/L2 = 2:1). These results support the importance of the hydrophobic tract between prolines 413 and 419 in HPV11 L1-L2 binding. Specifically, introduction of two negatively charged residues at positions 417 and 418 decreased L1-L2 binding.

Another feature of the sequence between residues 413 and 419 is the presence of three proline residues. All other L2 proteins examined in our studies also contain three prolines in this region with the exception of BPV1 L2, which contains two prolines. To test the importance of prolines 413, 416, and 419 of HPV11 L2 in L1 binding, each of these prolines was changed individually to alanine (P413A, P416A, and P419A), and the ability of these mutant L2 proteins to bind L1 was examined in coexpression assays. As shown in Fig. 7, lanes 5, 6, and 7, substituting an alanine for a proline did not change the ratio of L1 to L2 in all three cases, indicating that these mutations did not disrupt L1 binding.

Biochemical characterization of L1-L2 binding. Inspection of the carboxy-terminal L1 binding domain of HPV11 L2 (Fig. 5, top sequence) revealed a high percentage of hydrophobic residues (>50%). The results of our mutational analysis suggested that hydrophobic interactions are important in L1+L2 complex formation. To test the importance of hydrophobic interactions, we analyzed the ability of salt, weak detergents, and urea to disrupt the complex. A complex of HPV11 L1 bound to HPV11 GST=L2(313-455) was immobilized on glutathione-Sepharose beads and incubated with a series of buffers. The amount of L1 in each sample was determined by densitometric scanning of Coomassie blue-stained gels relative to samples treated with buffer L alone, representing the background level of L1 liberated from the complex (baseline), and samples treated with SDS-PAGE loading buffer, representing all bound proteins (100%). A typical result is depicted in Fig. 8A. The relative amount of L1 released by each buffer treatment averaged from three independent experiments is shown in Fig. 8B. Treatment with high salt concentrations (1 or 2 M; Fig. 8A, lanes 1 and 2), weak detergents (lanes 3 to 5), and urea (up to 2 M; lanes 6 to 9) did not release significant amounts (>20%) of L1 from the complex. Even treatment with 5 M urea failed to completely disrupt the L1+L2 complex (Fig. 8A, lane 11). Taken together, these results indicate that the L1-L2 binding is strong and likely mediated by hydrophobic interactions. These results parallel the findings for polyomavirus VP1-VP2 binding, for which the atomic structure of the binding interface reveals predominantly hydrophobic interactions (8).

L2 affects in vitro assembly of VLPs. The ability to purify L1 pentamers bound to L2 permits study of the specific effects of the minor capsid protein on in vitro assembly of VLPs. We examined the in vitro assembly properties of HPV11 L1 alone in comparison to a HPV11 L1+L2 complex. An L1+GST=L2 (313-455) complex was treated with thrombin to remove the GST moiety, and the resulting L1+L2(313-455) complex was dialyzed into assembly buffer at pH 5.2 or 6.8 in parallel with purified L1 as described in Materials and Methods. Acidic pH (5.2 to 5.4) was demonstrated previously to yield the most consistent in vitro assembly results in the case of both HPV11 L1 and HPV16 L1 (6). At pH 5.2, L1 alone assembled into either T=1 or T=7 VLPs (6), whereas L1+L2(313-455) assembled into T=1 VLPs (data not shown). At pH 6.8, L1 alone

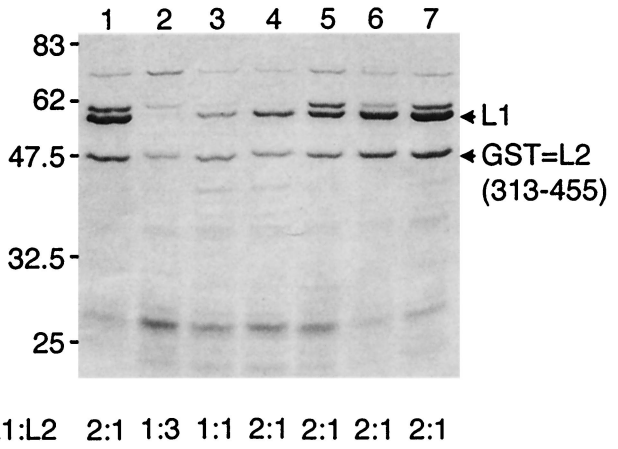

FIG. 7. Disruption of HPV11 L1 binding in site-directed mutants of HPV11 L2. A total of 10 μg of reduced glutathione eluates from coexpressions with wild-type GST=L2(313-455) (lane 1) and GST=L2 (313-455) carrying the mutations AL417418EE, A417E, L418E, P413A, P416A, and P419A (lanes 2 through 7, respectively) were separated by SDS-PAGE and stained with Coomassie blue. The ratio of L1 to L2, indicated below each lane, was determined by densitometry analysis. Note the difference in the ratio of L1 to L2 in lane 1 compared to that in lane 2.

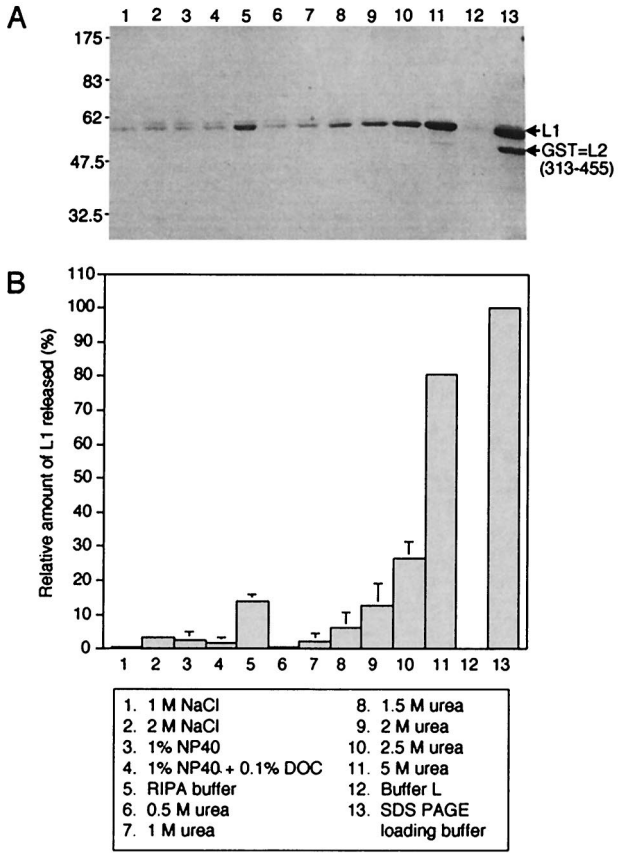

FIG. 8. Stability of L1+L2 complexes. An immobilized complex of HPV11 L1 and HPV11 GST=L2(313-455) was treated with the following: lane 1, 1 M NaCl in buffer L (40 mM Tris [pH 8.0], 200 mM NaCl, 2 mM DTT, 1 mM EDTA); lane 2, 2 M NaCl in buffer L; lane 3, 1% NP-40 in buffer L; lane 4, 1% NP-40 plus 0.1% DOC in buffer L; lane 5, radioimmunoprecipitation assay buffer (50 mM Tris [pH 7.2], 150 mM NaCl, 0.1% SDS, 1% NP-40, 1% DOC); lane 6, 0.5 M urea in buffer L; lane 7, 1 M urea in buffer L; lane 8, 1.5 M urea in buffer L; lane 9, 2 M urea in buffer L; lane 10, 2.5 M urea in buffer L; lane 11, 5 M urea in buffer L; lane 12, buffer L; and lane 13, SDS-PAGE loading buffer (63 mM Tris [pH 6.8], 10% glycerol, 2.3% SDS). A total of 50 μl of the supernatant from each treatment separated by SDS-PAGE and stained with Coomassie blue is shown in panel A. The positions of HPV11 L1 and HPV11 GST=L2(313-455) are indicated at the right. Molecular size markers are indicated at the left in kilodaltons. Panel B shows the relative amounts of L1 released from the complex by each treatment as determined by densitometry analysis of gels like that depicted in panel A. The bars correspond from left to right to the treatment indicated for lanes 1 to 13 in panel A. The values represent the average of three independent experiments.

was unable to assemble into VLPs, and free pentamers or aggregated clumps of pentamers were predominantly observed (Fig. 9). In contrast, L1+L2(313-455) assembled into T=1 VLPs at pH 6.8 (Fig. 9). These results suggest that L2 facilitates T=1 VLP assembly at a more physiological pH.

DISCUSSION

We utilized an in vivo bacterial coexpression system developed to study the VP1-VP2 interactions of mouse polyomavirus in order to study the intermolecular interactions of L1 and L2 of HPV11. Using bacterial coexpression, we defined a 44-amino-acid region near the carboxy terminus of L2 that specifically interacted with L1 with an estimated stoichiometry of one L2 molecule per five L1 molecules. L1 binding to this domain is mediated by strong hydrophobic interactions involving L2 amino acids 413 to 419. The strength of L1 binding by this domain under physiological salt concentrations implicates it as the principal domain responsible for anchoring L2 within the papillomavirus virion. Other weaker interactions with L1, such as those detectable under higher salt concentrations (37, 45), may serve to further align L2 within the virion and/or aid in encapsidation of the viral genome (37).

A striking feature of the 44-amino-acid L1-binding domain of HPV11 L2 is the presence of a proline at every third residue (PxxP), commencing at proline 410 (see Fig. 5). Proline-rich sequences containing a PxxP motif are commonly involved in protein-protein interactions, for example, in the ligands of proteins with Src homology 3 domains (29). Such an arrangement of prolines would also allow a sharp bending of the polypeptide chain, which may facilitate a short stretch of L2 to enter and exit the central cavity of the L1 pentamer.

The salt concentration used in our purification buffers was 0.2 M NaCl, and thus the domain we identified may correspond to the "salt-independent" L1-binding domain of L2 previously identified by Sapp et al. for HPV33 L1-L2 interactions (45). In these studies, HPV33 L1+L2 VLPs, purified after recombinant capsid protein coexpression in insect cells, were disassembled under low-salt (0.15 M NaCl) and high-salt (0.5 M NaCl) conditions. L1-L2 interactions that were detectable after low-salt disassembly were referred to as salt independent, whereas those that were detectable only after high-salt disassembly were referred to as salt dependent. For BPV1, a single 77-amino-acid L1-binding domain located near the carboxy terminus of L2 was identified by mixing disassembled L1 VLPs with in vitro-translated L2 under low-salt concentrations (37). This domain is analogous to the L1-binding domain identified

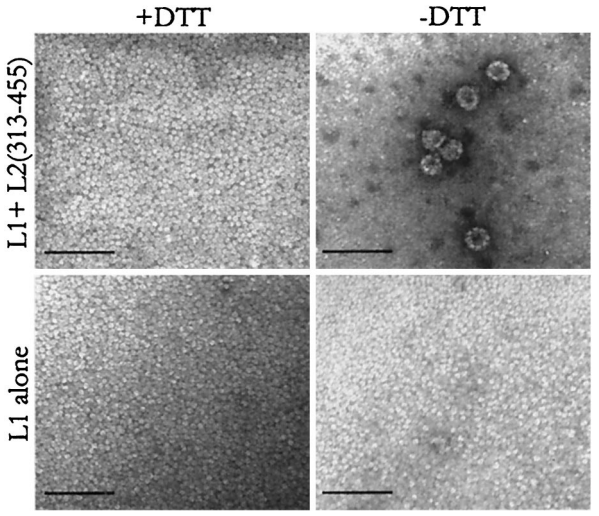

FIG. 9. An L1+L2 complex assembles at pH 6.8. HPV11 L1 alone or in complex with HPV11 L2(313-455) was dialyzed against 40 mM HEPES (pH 6.8) and 0.5 M NaCl in the presence or absence of 5 mM DTT. Representative fields are shown. The scale bars represent 100 nm.

in our studies with HPV11. An additional L1-binding domain was identified in the central portion of BPV1 L2 by using in vivo coimmunoprecipitation experiments carried out in buffer containing 0.5 M NaCl (37). Thus, this second L1-binding domain may represent a salt-dependent binding domain. A comparable second binding domain was not detected under our assay conditions.

Our initial investigations uncovered a strong influence of L2 on in vitro capsid assembly. When L1 is in complex with the carboxy-terminal 143 amino acids of L2, T=1 VLPs can form at neutral pH. Perhaps the carboxy terminus of L2 neutralizes charges on L1 and/or induces conformational changes in L1, thereby permitting the assembly of T=1 VLPs at neutral pH. The assembly of L1+L2 into full-size T=7 VLPs at neutral pH may require further modification of the in vitro assembly buffer conditions, different lengths of L2 or a combination of L1 and L1+L2 containing capsomeres.

The location of the principal L1-binding site on L2 and the stoichiometry and hydrophobic nature of the L1-L2 interactions have interesting parallels to the mouse polyomavirus VP1-VP2 interface (3, 8). However, although the major protein-minor protein interactions for the two virus families are similar in these respects, a major difference lies in the number of capsomeres occupied by the minor structural protein(s) within the virion. In polyomavirus virions, every capsomere is occupied by either VP2 or VP3, an internally initiated form of VP2 that shares the same carboxy terminus (18, 32). In papillomavirus virions there is a single minor structural protein, and only the pentavalent capsomeres are thought to be occupied by the minor structural protein (50). This difference in virion architecture may reflect the differences in the biology of assembly of these two viruses. Polyomavirus VP1-VP2/3 complexes assemble in the cytoplasm and are translocated together to the nucleus (12, 17, 25). Recent studies indicate that papillomavirus L1 and L2 do not preassemble in the cytoplasm but instead are separately translocated to the nucleus (16). L2 translocates to the nucleus first, localizes to promyelocytic leukemia oncogenic domains, and then recruits L1 pentamers to these domains for subsequent virion assembly (11, 16). These observations are consistent with L2 playing a direct role in facilitating the assembly of L1 pentamers into virions. However, thus far we have been unable to form complexes of L1+L2 in vitro without coexpression, suggesting that other mediating factors exist in vivo.

One practical application of our bacterial coexpression technology is in the improvement of both prophylactic and therapeutic papillomavirus vaccines for controlling cervical cancer. Prophylactic vaccines currently in clinical trials are based upon VLPs assembled from HPV16 L1 (5, 19, 23, 47, 48). Cross-protection against other papillomavirus serotypes may not be adequately provided by these VLPs, since neutralizing immune responses tend to be predominantly type specific (9, 10, 40, 41, 43, 51). L2 has been demonstrated to generate cross-neutralizing antisera (42), and thus inclusion of L2 in a vaccine formulation may provide a broader spectrum of prophylaxis than L1 alone. We have demonstrated in the present study that heterologous L1+L2 complexes can be produced in bacteria. Thus, it may be possible to develop heterologous VLPs or heterologous capsomeres, a promising alternative to VLPs (53), to further improve the spectrum of protection in a prophylactic vaccine. One approach to developing a therapeutic vaccine for treating established cervical carcinomas has been to create VLPs containing an immunotherapeutic protein, typically E7 ("chimeric" VLPs [36]). The L1-binding domain of L2 could be fused to the desired immunotherapeutic protein, thereby allowing its incorporation into a VLP. This approach offers certain advantages over strategies that utilized full-length L2 to incorporate the desired foreign sequence in vivo (20). Since only a portion of L2 need be used (possibly as small as 44 amino acids), longer foreign sequences can be introduced. Also, since every pentamer would have foreign sequence attached, it may be possible to incorporate 72 copies of the foreign sequence into a VLP. These features, coupled with the cost-effectiveness of a bacterial system, make this an appealing technology for chimeric VLP production.

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